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The emerging ability to manipulate matter at the molecular and atomic levels has allowed materials scientists to create an array of potential new commercial products. These manufactured nanomaterials can possess novel chemical and physical properties that cannot be predicted based on our current understanding of their behavior in large bulk forms. The potential for manufactured nanomaterials to interact with biological systems in adverse ways is attracting intense interest in the toxicology community, and studies are beginning to appear in the literature addressing health and safety issues associated with nanotechnology. The unique chemical and physical properties of nanomaterials present special challenges to the toxicologist attempting to design studies to accurately and reproducibly identify adverse biological interactions.

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Final Report

Developing Experimental Approaches for the Evaluation of Toxicological Interactions of Nanoscale Materials

A workshop addressing the challenges of conducting and interpreting studies of potential toxic effects of nanoscale materials

NOVEMBER 3 - 4, 2004

UNIVERSITY OF FLORIDA HOTEL AND CONFERENCE CENTER
GAINESVILLE, FLORIDA

Sponsored by:

US Department of Health and Human Services National Toxicology Program
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Preface

The workshop was organized by staff from the National Institute of Environmental Health Sciences and the University of Florida. Steering committee members participated in the development of the agenda and breakout group session topics, and in selection of invited attendees. This report was compiled and edited by the workshop organizing committee and reflects the deliberations and recommendations that emerged during the workshop and not the views of the workshop sponsors.

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Workshop summary

The emerging ability to manipulate matter at the molecular and atomic levels has allowed materials scientists to create an array of potential new commercial products. These manufactured nanomaterials can possess novel chemical and physical properties that cannot be predicted based on our current understanding of their behavior in larger bulk forms. The potential for manufactured nanomaterials to interact with biological systems in adverse ways is attracting intense interest in the toxicology community, and studies are beginning to appear in the literature addressing health and safety issues associated with nanotechnology.

The unique chemical and physical properties of nanomaterials present special challenges to the toxicologist attempting to design studies to accurately and reproducibly identify adverse biological interactions. Experimentation in this area is greatly complicated by a several issues, such as: a) dosimetry - how to properly express and/or administer the dose of nanomaterials (e.g., mass, dimension, surface area, surface coating, aggregation state); b) confirmation that the material given to the animal or cell culture is in the desired form; c) difficulty detecting and quantifying nanomaterials in cells and tissues; and d) the need to characterize nanomaterials in all stages of toxicological testing. Based on concerns that early literature reports of toxicology studies with nanomaterials may be unreplicable and perhaps uninterpretable, investigators at the University of Florida and the National Institute of Environmental Health Sciences organized a workshop in Gainesville, Florida, on November 3 and 4, 2004. This workshop brought together toxicologists, chemists, physicists, materials scientists and others to discuss the special challenges to the proper conduct and reporting of studies in the emerging field of "nanotoxicology."

The 75 invited participants represented expertise in biology, medicine, toxicology, physics, chemistry, and materials science drawn from government, industry, academic and public interest sectors. The participants heard presentations and addressed in breakout sessions; a) characterization and dosimetry of nanoscale materials, b) delivery of nanoscale materials to test systems, c) toxicology study protocols appropriate for nanoscale materials, d) detection and quantification of nanoscale materials in test systems/organisms and the environment, e) laboratory safety and disposal issues, and f) specific issues related to uptake and toxicity to the respiratory, skin, and immune systems.

Over the course of the two-day workshop, several central themes emerged from the presentations and discussions:

- It is essential that the physical and chemical characterization of nanoscale materials be much more complete than has been the case in the sparse toxicology literature appearing to date. State of the art analytical characterization techniques were described and their application to all phases of toxicology studies was considered. The use of currently available analytical techniques to detect and quantify nanoscale structures in biological systems was considered critical for both guiding the selection of the specific toxic endpoints of interest, and for following the movement of nanoscale materials in biological systems. The group recommended that scientific journal editors be urged to

require proper physical and chemical characterization of nanoscale materials for all publications in the newly emerging field of "nanotoxicology".

- Most participants agreed that "nanotoxicology" need not be a new scientific discipline. Based on our current understanding, the traditional approaches and study protocols now used for routine toxicological characterization of chemicals or larger particles are sufficiently robust to provide meaningful toxicological characterization of nanoscale materials. While nanoscale materials clearly have unique physical and chemical properties that may lead to unpredictable distribution and effects within biological systems, there was general agreement that the manifestation of biological interactions of nanoscale materials will likely be the same as for any potentially hazardous agent. The participants recognized that more suitable approaches for nanoscale material characterization, detection and/or toxicological evaluations may emerge with time and experience.

- Participants stressed the need to approach nanotoxicology studies from a multidisciplinary approach and recommended that government agencies explore ways to create and promote linkages between toxicologists and experts in materials science, physics, chemistry and other appropriate disciplines. Government agencies were also asked to provide assistance with the creation of standard reference materials, and in the development of accreditation programs for analytical laboratories engaged in the analysis and characterization of nanoscale materials.

All participants recognized the tremendous potential of nanotechnology to provide benefits to society. There was also the recognition that the toxicology community needs to take best advantage of the time remaining before human exposures to manufactured nanomaterials become widespread. The studies done now must be accurate and reliable to help ensure the design and development of nanomaterials that are benign to human health and the environment.

Introduction

With the explosive growth of nanotechnology, questions have arisen whether the use of new nanoscale materials might have unintended human health and environmental consequences. Studies of biological effects of nanoscale materials that might answer these questions have lagged behind other aspects of nanotechnology development. There is widespread interest in addressing this information need, but conducting studies of biological interactions of nanoscale materials poses several challenges. Issues regarding safe handling of potentially toxic nanoscale materials by researchers have not been well worked out. Also, toxicity characterization of test materials is more complex than with conventional chemical studies because it involves, in addition to composition, aspects of size, shape, and surface properties. There are questions of proper dosimetry and potential problems in detecting some nanostructures in biological tissues. As investigations of biological effects of nanostructures move forward, it will be important to reach consensus on requirements for safe use of nanomaterials, characterization of test materials needed such that studies can be correctly interpreted and reproduced, and ways in which experimental designs should be modified to address special issues associated with nanostructures. During this workshop, investigators in the field addressed the need for developing best experimental practices for studies of potential toxicity of nanoscale materials.

The two-day workshop was organized into a half-day plenary session followed by two breakout sessions to address specific issues in smaller group discussions. The results of these breakout sessions were then compiled and discussed in a final plenary session on Day 2. Four nationally recognized leaders in their respective fields delivered plenary session presentations. Dr. Clayton Teague, Director of the National Nanotechnology Coordination Office was the first speaker and provided a national perspective on nanotechnology and potential toxicity issues. He was followed by Dr. Brij Moudgil, Director of the National Science Foundation Particle Engineering Research Center at the University of Florida. Dr. Moudgil discussed issues and challenges in characterizing nanoscale materials used in toxicity studies. The third speaker was Dr. Gunter Oberdorster from the University of Rochester, an internationally recognized expert on the health effects of ultrafine particles and director of one of the U.S. Environmental Protection Agency's Airborne Particulate Matter Centers. Dr. Oberdorster's presentation covered issues of proper dosimetry of nanoscale materials and challenges in characterizing the fate of nanoscale materials in the body. The final speaker was Dr. Michael Luster from the National Institute for Occupational Safety and Health. Dr. Luster discussed issues associated with conducting toxicity tests using nanoscale materials. The Workshop Program and slides from these plenary presentations can be found on the University of Florida's nanotoxicology website at www.nanotoxicology.ufl.edu.

The topics for the breakout sessions were developed in cooperation with the Workshop Steering Committee. There were ten breakout groups assigned to address the following nine topics:

Group 1: Characterization of Nanoscale Materials
Group 2: Dosimetry of Nanoscale Materials
Group 3: Nanoscale Materials Testing Protocols – Group A
Group 4: Nanoscale Materials Testing Protocols – Group B
Group 5: Exposure Protocols: Practical Issues

Group 6: Issues in Assessing Potential Immune System Effects
Group 7: Issues in Assessing Pulmonary Intake and Toxicity of Nanoscale Materials
Group 8: Issues in Assessing Dermal Uptake and Toxicity of Nanoscale Materials
Group 9: Detection and Quantification of Nanoscale Materials in Toxicity Studies
Group 10: Laboratory Safety and Disposal Issues

Specific questions were developed to guide the discussions, but groups were free to add or adjust the scope of the discussion as desired. This flexibility led to some drift in discussions, resulting in some overlap. No attempt was made to prevent this. Rather, the fact that different groups, addressing different topics felt the need to address, for example, the importance of characterization of test materials reinforced the significance of that aspect of nanotoxicology studies. A moderator was assigned to lead each breakout group, as well as a recorder to capture the discussion, findings and recommendations. During the plenary session on the second day, each moderator reported the findings to all of the workshop participants and the floor was opened for discussion.

After the workshop, brief summary reports for each breakout group session were prepared by the moderators, recorders, and organizing committee, synthesizing the breakout group discussions, as well as comments and issues that arose during the second day plenary session. Based on these summary reports, the organizing committee prepared this final workshop report.

Major Findings and Recommendations

Major Findings

- It is essential that the physical and chemical characterization of both bulk and formulated test nanoscale materials be much more complete than has been the case in the sparse toxicology literature appearing to date.
- Most participants agreed that while nanoscale materials have unique chemical and physical properties, the manifestation of biological interactions of nanoscale materials will likely be the same as for any other potentially hazardous agent. However, careful selection of appropriate toxicological test methodologies is necessary.
- Participants stressed the need to approach nanotoxicology studies from a multidisciplinary team approach.

Workshop Recommendations

- In order to identify the key aspects of the physical/chemical nature of nanomaterials that are related to their potential toxicological effects, a minimum set of relevant nanomaterial characteristics should be developed for toxicological studies.

- Given the need for appropriate characterization of nanomaterials, a national network of facilities specializing in the characterization of nanoparticulate materials for nanotoxicology studies should be established. The purpose of this network will be to support and facilitate the research of toxicologists working in this field. Standards organizations (e.g., National Institute of Standards and Technology and the American National Standards Institute) should be involved.

- Evaluation of the safety of nanomaterials should be primarily based on traditional *in vivo* toxicity models rather than use of *in vitro* assays. However, in addition to these established *in vivo* methods, expanded use of *in vitro* assays and molecular/mechanistic endpoints should be employed in initial evaluations of current nanomaterials to facilitate development of an appropriate *in vitro/in vivo* tiered testing paradigm that can be applied to future developed nanomaterials.

- There is a need for research determining key aspects that determine the absorption, distribution, metabolism and elimination (ADME) of nanomaterials in biological systems, and for the development of mathematical models that may be useful in helping predict the ADME and or biological interactions of nanoscale materials.

- The organizers should consider disseminating the results of this workshop to the nanotechnology community through professional publications and trade journals.

- Priority should be given to developing safety guidelines and recommended practices for scientists conducting research in this area.

- Smaller, more focused workshops should be organized to address the key specific issues in greater detail. These workshops can be conducted in person, by teleconference or other means of communication. Each would compile a list of recommended practices in their specific area. These would then be disseminated to the research community.
- A future national workshop should be organized to discuss the progress and results of these focus groups and to continue planning a national strategy for nanotoxicology studies.

Breakout Group Discussions

Group 1: Characterization of Nanoscale Materials

Brij Moudgil, University of Florida *(Moderator)*
Ben Koopman, University of Florida *(Recorder)*

Is it necessary to characterize nanoscale materials as provided by the manufacturer, as delivered in the dosing formulation, as it exists in the biological environment (in vitro or in vivo), or some combination of these? What descriptors are important (e.g., structure size, shape, density, surface chemistry, etc.)?

The group agreed that nanoscale materials should be characterized as they exist in the biological environment. However, certain characteristics of the materials as received from the manufacturer and as dosed should also be cataloged for quality control purposes. Some participants pointed out that "nameplate" specifications given by manufacturers are not always met. Furthermore, characterization methods used by the manufacturers are usually not specified.

A baseline suite of parameters should be identified, with the understanding that this suite would most likely be both material and application specific. These parameters could generally be categorized by whether they are determined *ex vivo* or *in vivo*, and more specifically categorized according to whether they describe physical or chemical characteristics. The list of candidate parameters is given below.

Ex vivo

- Physical: size, shape, surface area, surface porosity, roughness, morphology (agglomerate vs. primary particles, stability of agglomerates), crystallinity, magnetic properties
- Chemical: stability (dissolution), chemical composition, surface chemistry [zeta potential, acidity/basicity, redox potential, functional groups, reactivity (catalysis, redox, photosensitivity)]

In vivo

- Images, dispersibility, dosage (number density for materials with narrow size distribution; mass dosage for materials with wide size distribution)

With regard to the *ex vivo* characterization, it was noted that the distributions of particle properties should be considered, in addition to statistics of central tendency such as the mean or median. Also, the history of the particles should be considered as a means of determining what is likely to be on the surface, e.g., chemical functional groups. Thus, reference to the core synthesis paper should be included when reporting particle properties along with some characteristics regarding stability and aging. In the determination of *in vivo* dosing, radioactively labeling particles (e.g., with tritium) should be considered wherever possible, but investigators must be cognizant of possible effects of labeling on particle properties.

What methods should be used to characterize nanoscale materials?

The group separated these methods according to whether ensembles of particles or single particles were subject to the characterization method. The large majority of applicable characterization techniques are ensemble techniques. In making these measurements, the biological media must be considered.

Measurement of the size of most nanoparticulate systems can be adequately carried out using light scattering or other techniques. Smaller particles should be imaged in order to assess their size and shape. Even larger particles should be imaged for comprehensive characterization. Transmission electron microscopy (TEM) is applicable to electron dense material in the nano size range. Cryogenic TEM (or more conveniently, scanning TEM) provides information for particle size distribution, mean size, and concentration. Public domain software is preferable for computing statistics of distribution. Caution must be employed in interpreting the TEM results, and a statistically valid number of particles must be counted.

TEM should not be the sole instrument used for measuring particle size. Other techniques that have been used with success are mass spectrometry, analytical ultracentrifugation, size exclusion chromatography, capillary electrophoresis, and gel electrophoresis.

The length scale must be considered in selecting appropriate techniques for surface area measurement. A logical starting point is the protein length scale. Applicable techniques are gas adsorption, differential mobility analysis for aerosols, and potentiometric titration.

Techniques for assessing surface chemistry of particle ensembles include X-ray photon spectroscopy, Atomic Force Microscopy-based scanning probe techniques, and bio Raman. Elemental composition of particle ensembles can be determined by energy dispersive X-ray spectroscopy, as well as other standard techniques such as digestion and inductively coupled plasma analysis.

The group agreed that "wet side" techniques developed by biologists should be taken advantage of to supplement or, where appropriate, replace the classic materials science "dry side" techniques. Finally, the one single particle method mentioned was electron energy loss spectroscopy.

What is the minimum description of a nanostructure necessary in a scientific report of the research?

The group decided that the elemental composition of the particles is a must and should include a reference to the core synthesis paper. In addition, surface morphology, degree of crystallinity, and imaging by TEM were considered necessary.

Would it be worthwhile for NIST to produce a set of reference nanomaterials?

This question elicited considerable discussion. It was mentioned that this would be helpful for toxicologists not actively collaborating with materials scientists or with limited characterization resources. Should NIST be interested, it should recognize that quick turnaround would be important to the scientific community to avoid missing the window of opportunity for helping nanotoxicity investigations. NIST reference materials are currently used to validate NIST-traceable facilities. Hence, provision of the reference nanomaterials could help alleviate the current situation in which there are few commercial laboratories with broad nanomaterial characterization capabilities.

One concern brought up in this discussion was that the availability of reference nanomaterials might lessen the need for collaboration between materials scientists and toxicologists. However, substantial benefits would accrue to the science of nanotoxicology, to commercial nanoparticle characterization laboratories, and to manufacturers of characterization equipment.

What are mechanisms for disseminating our recommendations to the scientific community?

Editorials by individual scientists or an open letter from several members of the nanotoxicology and particle science communities were identified as feasible mechanisms of dissemination. It was suggested that recommendations originating from a recognized group, such as the participants in this Workshop, might be more credible than recommendations made by an individual.

Group 2: Dosimetry of Nanoscale Materials

Paul Howard, National Center for Toxicological Research/US FDA *(Moderator)*
Sally Tinkle, National Institute of Environmental Health Sciences *(Recorder)*

How should doses of nanoscale materials be expressed? Should they be expressed in terms of surface area, mass, volume, particle number, or some other property?

The group decided that, depending on the circumstances, surface area, mass, volume, particle number, or some other property could be the appropriate basis for expressing the dose of a nanoscale material. The nature of the material itself could be the most important determinant of this decision. The overriding concern is that good science should be conducted for any toxicological study. In this regard, studies of nanoscale materials should not differ from studies of other materials with potentially toxic effects. The approach taken by the group was to envision guidelines that would be desirable for journal editors and reviewers of journals, grants, and other scientific communications.

Characterization (and reporting) of the material to be used is crucial to dosimetry. The group agreed that the characterization of a nanomaterial should include:

- Description of preparation methods and identification of the material with conventional analyses
- Assessment of purity (This might be from a NIST certified laboratory, or using a NIST certified method.)
- Particle size distribution on the basis of volume and mass, number of particles per unit volume or mass (mL or g), surface area, and particle shape
- Characterization of stability
    a. Description of methodology, e.g., "1 g was suspended in 4 mL of acetonitrile, vortexed, and analyzed using a ..."
    b. Estimation of the half-life of chemical stability and aggregation
- Shape and crystal structure, if appropriate
- Surface charge

As part of the discussion of materials characterization, the group noted that archiving of a test material would give investigators the capability to re-examine a material should the need arise or to apply new or different methods of characterization. However, the group was uncertain how this would work if the material were proprietary. Who would do the archiving?

The expertise and resources required for adequate characterization of nanomaterials are a significant concern. It was argued that a diverse team approach is called for, in effect sharing the burden among groups. Groups that wish to carry out the full menu of needed characterizations themselves should adequately budget for this task.

Once the nanomaterial is properly characterized, the appropriate dosimetry may be addressed. Points brought out in the group discussion were:

Inhalation toxicology studies. Standard study designs are sufficient to assess nanoscale materials. However, investigators must be aware of possible unique issues of nanoparticle behavior in generating aerosols for inhalation studies.

Dose. How should it be reported, e.g., particles/10^6 cells; molarity of target drug (inside nanoshell); etc? Regardless of the basis chosen, sufficient information should be available to convert the dose to different units if adequate characterization information is included in the description of the material. A related discussion point was the importance of dosimetry at the target organ. Appropriate methodology for target organ dose assessment is dependent on the absorption, distribution, metabolism, and elimination (ADME) of the material.

Characterization of test material. "Beginning and end" - What material characteristics did you start with and what were the characteristics when the material was applied to your test platform? In order to answer this question, the investigator must characterize the stability and distribution of the test material at the time of application/use. This would include:

- Full description of vehicle, media, mixing methods, etc.
- Characterization of aggregation, surface charges (if appropriate)

- Characterization of what is within the cell, if possible

Group 3: Nanoscale Materials Testing Protocols – Group A

Nigel Walker, National Institute of Environmental Health Sciences *(Moderator)*
Phil Sayre, United States Environmental Protection Agency *(Recorder)*

Are there hazards of special concern for nanoscale materials that suggest a need to modify traditional toxicology testing protocols? If such hazards exist, which protocols should be modified, and in what way? What basic research is needed to guide the modifications?

In general there was consensus that while nanoscale materials clearly represent a unique class of materials that have many novel and unique physiochemical properties and biological interactions, it is too early to define what "new hazards" there may be. The issue discussed was whether these unique biological properties would be manifested in novel toxicities. It was generally felt that this was unlikely, especially with regard to the development of pathological endpoints that may be considered hazardous. Biological systems have the capacity to integrate multiple mechanisms of action of diverse hazardous agents, and these mechanism tend to be manifested at the cell and tissue level in similar ways that are considered to be toxicological responses. Examples include proliferation, apoptosis, necrosis, hyperplasia, hypertrophy, metaplasia fibrosis, and carcinogenesis.

What endpoints (biochemical, genetic, or morphological changes) would be most appropriate for conducting screening tests (e.g., in vitro) for toxicity of nanoscale materials? Are there special issues in extrapolation of toxicity testing (from in vitro or animal tests) with nanoscale materials for use in human health risk assessment (different from chemicals, for example)?

Current procedures and guidelines used to detect toxicological responses such as those recommended by OECD/EPA/FDA likely would be appropriate. However, concern was raised that selection of the most appropriate test to use *a priori* is an area that requires special consideration, especially for nanoscale materials. This is due to the fact that, in the literature of nanomaterial deposition/absorption in the nasal cavity, the expected route of exposure adsorption, distribution, metabolism and elimination (ADME) of nanoscale materials (NSMs) may be unpredictable. This may be due to the fact that ADME occurs at the interface between molecules and particles, and as such, nanoscale behavior may lie somewhere in between the two, with a blended characteristic depending on the material size/chemical composition. Therefore, the need to carefully characterize the materials under evaluation both before and during testing was deemed to be of utmost importance. In addition, the material and dose formulation characterization, and evaluation of the ADME of NSMs, were felt to be critical in terms of guiding selection of the type of test to be used. As an example, it may guide more in depth pathological evaluations in specific tissues or prompt adjunct mechanistic endpoint evaluations in specific tissues.

Short-term *in vitro*/*in vivo* tests should be considered to provide additional information on model selection, but as yet cannot be a replacement for the *in vivo* tests or be used for hazard ranking.

There is a need to build a knowledge base that allows us to investigate how the unique properties of NSMs are related to potentially pathological response and hazard. Given the uncertainty in correlation between specific *in vitro* endpoint data and established hazardous responses, care needs to be exercised in the potential misinterpretation of the human health significance of *in vitro* endpoints evaluated for NSMs.

In addition, research is specifically needed in the area of determining key aspects that determine the ADME of NSMs. It was suggested that specific efforts be made in the area of development of quantitative structure-activity relationships (QSAR) and physiologically-based pharmacokinetic (PBPK) models that may be useful in helping predict the ADME and/or biological interactions of NSMs, such that better decisions can be made for development of the most appropriate test group.

Given the ability of NSMs to aggregate, concern was raised about generalization of conclusions from one battery of assays. Reproducibility across labs and /or models may be less consistent. This again reiterated the need for good characterization of what was actually tested. To this end, it was suggested that there be a repository of materials tested such that post-hoc analyses could be conducted should a given dataset be used for a critical regulatory decision. The problem of stability of the NSM needs to be considered. The use of reference materials for such analyses may be of utility, although the issue of needing a "standard standard " was noted.

An unresolved issue that pertains not only to NSMs but any potentially hazardous agent is the selection of the most appropriate battery of tests, given that evaluation in every possible assay of toxicological significance is unrealistic.

Group 4: Nanoscale Materials Testing Protocols – Group B

Scott Masten, National Institute of Environmental Health Sciences *(Moderator)*
Barbara Karn, United States Environmental Protection Agency *(Recorder)*

Are there hazards of special concern for nanoscale materials that suggest a need to modify traditional toxicology testing protocols? If such hazards exist, which protocols should be modified, and in what way? What basic research is needed to guide the modifications?

The group concluded that at present there were no special hazards of concern for NSMs and that the toxicological manifestations of exposure to NSMs will likely be qualitatively similar to other hazardous agents. Any modifications to existing toxicological test methods to address potential hazards would be of a supplementary nature, and implemented incrementally over time as our understanding in this field evolves.

What endpoints (biochemical, genetic, or morphological changes) would be most appropriate for conducting screening tests (e.g., in vitro) for toxicity of nanoscale materials?

The group attempted to address, in broad terms, how one would design a research and testing program to characterize potential hazards of NSMs. In this context, broad means considering all

routes of exposure (e.g., inhalation, dermal, oral, ocular), all physical-chemical forms (e.g., solid, liquid) and effects on multiple organ systems (beyond portal of entry).

The substantial costs of generating and procuring NSMs argues for a tiered testing approach in which there is a role for both *in vitro* and *in vivo* methods, as well as comparative studies with bulk scale "inerts" and "toxics". It would be useful to have a large public database (e.g., a public data depository) in two species using conventional models and test methods such that pooled data analyses could be performed and be related to other historical test data (chemicals, particles). A methods "toolbox" needs to be sufficiently robust to assess the diverse range of anticipated NSMs. There is much to be learned, however, from replicate controlled studies on well-characterized standard materials (e.g., structural alerts may emerge).

During plenary discussion of the breakout group reports, it was acknowledged that tiered testing strategies must be both pragmatic and science-based, such that early tier studies are truly predictive and not just a logical sequence. If possible, it may be useful to develop a formal decision matrix for a tiered testing approach.

The group considered it critical to first adequately characterize the test material with respect to purity (e.g., metal content, endotoxins), solubility, porosity, roughness, adsorptivity, aggregation/disaggregation potential, size distribution, shape/aspect ratio, surface area, surface charge, surface coating, surface reactivity, storage requirements, and aging.

It will also be important to ensure adequate availability of materials to test, either already adequately characterized or standard reference materials. Since the process of making standards moves too slowly, a central facility for physical-chemical characterization (such as the NCI's Nanotechnology Characterization Laboratory) is recommended. Development of guidance for which physical-chemical characteristics are most critical for specific types of studies (*in vitro* vs. *in vivo*), as well as technical specifications requirements, was also recommended.

Dosimetry will prove very important when performing toxicological characterizations of NSMs. Methods or guidance is needed for how to detect NSMs in biological systems to determine delivered dose, as well as what are the appropriate dose metrics that account for biopersistence. Methods must allow one to measure NSMs within tissues and cells in soluble, single, and agglomerated form. Existing particle dosimetry models (rat, human) may need to be modified to account for the unique aerodynamics of nanomaterials.

Early studies will need to focus on determining bioavailability, absorption, distribution, metabolism, and elimination (ADME), but there are many questions to be answered. ADME is influenced by route, but will new empirical data for all routes be necessary? Is gastrointestinal and pulmonary dissolution of aggregates similar? Are the assumptions in physiologically-based pharmacokinetic models for larger bulk materials valid for NSMs? Will measurement and analysis capability be limiting? Is radio labeling and tagging of NSMs acceptable for evaluating ADME of NSMs?

In considering appropriate test methods for NSMs, the group discussed what endpoints might not typically be measured in a toxicology test battery, recognizing that expansion may be needed,

and methods will evolve as more is learned on potential hazards. Screening assays based on intermediate biological responses would be useful if predictive, but must be representative of reality (i.e., anchored to *in vivo* correlates) and amenable to analyses that relate back to physical-chemical properties [for building QSARs]. Screening assays will also help inform evolutionary design of lowest toxicity or safe NSMs without impairing intended applications.

"-Omic" approaches will be useful for evaluating systemic changes that may be missed by biochemical endpoints, particularly as our understanding of the gene and protein expression changes associated with specific toxicological perturbations improves. These approaches are also amenable to short time courses.

Toxicological evaluations must focus on systemic, and not just local responses, such as:

- C-reactive protein, bronchoalveolar lavage fluid analyses, platelet aggregation and other immune and inflammatory responses [Are appropriate methods available to evaluate low level or weak inflammatory responses?]
- Oxidative stress, e.g., lipid peroxidation, tailored transgenic animal models
- Cytotoxicity (and its underlying mechanisms), blood cells (e.g., leakage of hemoglobin), GFAP (as a biomarker for neurotoxic insult)
- Genetic toxicity, e.g., are NSMs getting into cells/nucleus? Is there a difference in using bacterial or mammalian cells?

A final point related to the possible retrospective analysis of the existing toxicology database for ultrafine particles and fibers for identifying predictive endpoints. However, the lack of adequate physical-chemical characterization, and different methods of administration and test protocols used, may limit the utility of this approach.

Are there special issues in extrapolation of toxicity testing (from in vitro or animal tests) with nanoscale materials for use in human health risk assessment (different from chemicals, for example)?

With regard to special issues in extrapolation of toxicity testing results for use in human health risk assessment, the group was unable to judge whether this important issue will be any different than from chemicals or larger particles. It is still too early in the development of the field of nanotoxicology with too few available studies to draw meaningful conclusions as yet. It is clear, however, that dosimetry will be key. To permit appropriate extrapolation, toxicology studies must allow determination of whether adverse responses are driven by concentration or concentration x time, solubility, persistence, etc.

Group 5: Exposure Protocols: Practical Issues

John Schlager, United States Air Force *(Moderator)*
Greg Erdos, University of Florida *(Recorder)*

To what extent should aggregation of nanoscale materials be prevented or reversed in toxicity testing, both in vitro and in vivo?

The group noted that this was a materials handling question and that very different materials will be used in experimental studies. Storage conditions and aging are concerns for active/reactive materials. Stable polar materials could be dispersed mechanically. Nonpolar materials (e.g., naked, underivitized polycarbon) aggregate naturally in aqueous medium. Surfactants and chemical modification are alternate approaches to dispersion of these particles. However, changing particle characteristics could affect the tendency of these materials to enter cells, as well as their toxicity to cells. For example, aggregation of particles in the lung is associated with granulomas, whereas their dispersion is associated with inflammation. Also, if nanoparticles are coated with lung surfactant prior to cell model system exposure, the cellular fate differs from that of uncoated particles. These issues require the experimenter to consider closely their approach to answer specific questions on toxicity or resultant bioeffects.

The final decision to allow, prevent, or reverse nanoparticle aggregation during *in vivo* studies was considered by the group to depend on study design and the research questions being asked. The answer would be "no" or ("maybe") for *in vivo* experimentation where the intent is to model real occupational exposure or to mimic industrial or laboratory exposure conditions. On the other hand, if the intent of *in vivo* exposure is to understand the mechanism of a toxic response, the answer would be "yes" to capture mechanistic data. The preferred method of dispersion in this case is mechanical.

The group decided that prevention (or reversal) of aggregation is also preferable for *in vitro* experimentation to rapidly screen nanoscale materials or to develop mechanistic information. Multiple dose compositions are advisable. Researchers should attempt to prevent aggregation using tissue methods for coating/disaggregation of nanoparticles. These methods should be appropriate to the model phenotypic cells being studied. For example, coating the nanomaterials with lung surfactant would be appropriate if working with lung cells.

The need for standard (high-volume) nanoparticle materials from NIST was discussed. Examples of useful standard nanomaterials are TiO_2, SiO_2, fullerenes, single wall carbon nanotubes, and multiwall carbon nanotubes. These materials should be subjected to standard storage and handling procedures and come with explicit handling instructions for assuring sample characteristics are maintained during storage and use. They should also include appropriate procedures for dispersion. NIST should also articulate a set of principles for handling and testing the materials, as well as providing reference standards for interlaboratory comparison and repeatable studies.

Is testing with a wide distribution of particle sizes, or with material that exists primarily as large aggregates satisfactory?

Testing with a wide distribution of particle sizes or large aggregates could be warranted in the context of materials generated under occupational exposures (including laboratory sample handling). However, to build toxicology knowledge (e.g., mechanism-based toxicity studies related to size/quantum effects, with otherwise constant physicochemical conditions), particles

must be obtained in narrow size ranges at selected median sizes throughout the interval of interest. Behavior and associated toxicology of agglomerates in biological systems is important, but largely unknown. Thus, investigators should consider methods to follow the fate of aggregated particles and dynamics of disaggregation/reaggregation in biological models.

Should the approach be "whatever happens to them, happens" or should efforts be made to test with nanoscale materials that are in fact nanoscale and fairly uniform in size?

The "whatever happens to them, happens" approach is never good in basic experimental research. However, it may be appropriate for *in vivo* modeling of human exposure. Uniform nanoscale particles are necessary for mechanistic toxicity testing. The effects of nanoscale materials with large dimensional size ranges can be related to the effects of the same types of materials, but with uniform dimensions.

Nanoscale materials undoubtedly interact rapidly with macromolecules such as proteins in vivo. When conducting in vitro studies of nanoscale material effects, how important is it to duplicate these interactions?

If a surfactant coating occurs and is known, as in the case of lung surfactant, the group suggested that particles be preincubated with solutions of the appropriate proteins. It was noted that, in general, *in vitro* culture conditions are diverse and differ significantly from *in vivo* conditions in most aspects.

A basic research question is the extent and nature of nanomaterial-protein and protein-protein interactions. Most protein interactions are not well known. Based on available data, some interactions appear very selective. Researchers should consider post-determination analysis of proteins using techniques such as gel electrophoresis and proteolytic digestion-MALDI-mass spectrometry.

For example, could a cell culture experiment yield spurious results if a nanoscale material isn't presented to the cells with the same proteins on its surface as exists in vivo?

The answer to this question is "yes". Lung research studies prove that results are influenced by the nature of the protein coating on nanoscale materials.

Some general commentaries from the group discussions are as follows. First, concerns about exposure protocols are different depending on whether *in vitro* vs. *in vivo* studies are being carried out, and for *in vivo* studies, concerns differ depending on the target organ. Second, materials should be characterized throughout an experiment in order to account for the effects of particle aging and agglomeration. Also, basic mechanistic studies are needed to model nanoparticle behavior, as well as cell-nanomaterial interactions. Finally, a tiered testing strategy is recommended for evaluating the toxicity of nanoscale materials. This should include positive and negative reference samples and appropriate conditioning and handling of nanoscale materials prior to dosing. Testing should begin with cell-free assays. Target systems should then be selected and exposure and endpoints determined, followed by *in vitro* testing with single cell

models. Based on these results, materials for further testing using *in vivo* models can be selected and the dose-response relationships characterized.

Group 6: Issues in Assessing Potential Immune System Effects

James Baker, University of Michigan *(Moderator)*
Dori Germolec, National Institute of Environmental Health Sciences *(Recorder)*

What is the likelihood of immune recognition of nanomaterials?

The likelihood of immune recognition of nanomaterials is unknown. There is the possibility that because of the ability to interact with proteins, surface characteristics or potential catalytic capabilities, materials of this size would be uniquely recognized by the immune system as compared to similar materials of larger size. This might involve altered self-recognition of proteins that were denatured, or the recognition of molecular compounds from the nanomaterials. Another concern is that the alteration of proteins or biological membranes by nanomaterials will present altered structures that will appear foreign to the immune system and lead to an immune response.

What is the most likely path of exposure to the immune system?

General consensus was that inhalation is most likely to lead to immune system recognition of nanomaterials given the large surface area of the respiratory system, the large numbers of immune cells that interact with respiratory epithelial cells, and the documentation of this route as a means of sensitization to other types of similarly sized particles. However, there could also be differential recognition in tissues, such as the gut and the dermis, where nanomaterials are recognized after penetration through pores or hair follicles. Intravenous introduction is a potential route for immune recognition, but these types of exposures would likely be to medical or therapeutic preparations of nanomaterials. This approach may be important in terms of systemic dissemination of a material. Nanomaterials are thought to be transported, like antigens, by antigen-presenting cells to draining lymph nodes where an immune response could occur. This could certainly happen after exposure through any mucosal surface or in the vasculature. The interaction of these molecules with more general and non-specific immune receptors, such as the toll-like receptors, may activate inflammatory responses to a greater degree than specific immune recognition. Finally, the reticuloendothelial system will probably be the source of clearance of many of these materials given their size. While this may not lead to an immune response, it certainly raises issues concerning handling of this material by the reticuloendothelial system and elimination of this material from an organism.

What approaches might be attempted to prevent immune recognition from occurring?

Several issues were brought up. Certain types of covalent binding to surfaces such as polyethylene glycol may be useful in this regard. This approach has been suggested for a number of materials such as metal particles and quantum dots. However, the stability of these coatings and their longevity in materials that are retained in an individual must be fully evaluated

before this option is considered. Emulsifying this material into oil droplets or some other type of form that would carry it in the blood stream and then allow it to be filtered from the kidney is another option. Altering the structure of the material, i.e., providing biodegradable components to the nanomaterials may be very useful. Breakdown induced by low pH, by hydrolysis, by charge changes and other interactions such as oxygen free-radicals, may be very useful in degrading nanomaterials and preventing their recognition by the immune system. Another means of preventing immune recognition would be to target material to specific tissues or cells, thus blocking its recognition or exposure to the immune system. Finally, accelerated clearance of the material could also prevent its exposure to the immune system in a way that might prevent immunogenicity in the long-term.

With the increased use of nanoparticles for pharmaceutical applications comes the potential for adjuvant effects. Should an evaluation of immunogenicity be part of the safety assessment of nanomaterials?

It was felt that this should be a part of the initial evaluation, but could potentially be accomplished by standard QSAR techniques with evidence of inflammation evaluated via histopathology and standard toxicological studies.

In addition, what tools are available when immune effects are a concern in a biologic system?

The first thing would be to define the route of exposure to identify how the individual may be exposed to the material and to determine handling methodologies. In addition, an evaluation of parameters such as inflammatory mediators or hematologic screens, including acute phase proteins or C-reactive protein (CRP), would also be helpful for a general analysis of an immune response and in determining its severity. There are accepted models for contact allergy and lymph node proliferation studies that may be useful to determine sensitization or adjuvant effects of specific compounds. In addition, while animal models of type I allergy and autoimmune disease are well known, the predictive value of these models for human disease has not been fully validated.

Many of the coatings used in nanoparticles have been shown to be immunosuppressive. Is the traditional tiered testing approach used by the National Toxicology Program (NTP) sufficient to detect the immunomodulatory potential of nanomaterials?

It was felt that this testing was sufficient. If there were indications of immunomodulation from QSAR, standard toxicology studies, or studies of immune activation, then the current tiered testing panel should be sufficient to determine whether or not immunosuppressive effects are being mediated by nanomaterials.

Should additional endpoints not routinely used in screening (i.e., macrophage studies, reticuloendothelial system clearance studies) be added to the testing panel used for nanomaterials?

It is unclear to the group how the current testing panels predict other types of effects for nanomaterials, particularly blockade of the reticuloendothelial system and adjuvant activity.

Therefore, it is probably not warranted to introduce these tests at this point in time. However, the NTP should continue to evaluate these tests and employ them when needed for further evaluation of nanomaterials.

Group 7: Issues in Assessing Pulmonary Intake and Toxicity of Nanoscale Materials

David Warheit, Dupont *(Moderator)*
David Barber, University of Florida *(Recorder)*

What is a reasonable testing strategy for assessing the toxicity of inhaled nanoparticulates? Should a tiered testing approach be implemented?

The group concluded that a reasonable testing strategy could be organized to encompass a tiered testing approach in the following temporal sequence:

1) extensive particle characterization
2) pharmacokinetic/ADME studies (absorption, distribution, metabolism, excretion)
3) short-term intratracheal instillation studies
4) short-term inhalation studies
5) longer term inhalation studies

Additional comments that clarify or modify this approach include the following:

- Inclusion of acellular studies (e.g., ROS – reactive oxygen species, and other studies) is likely to provide information on mechanisms, particularly if toxicity is associated with the presence of catalysts.
- The expense and availability of test material may limit the number of *in vivo* inhalation studies.
- Concerns were raised regarding the relevance of the particle distribution characteristics (vs. aerosol exposures) associated with preparing and exposing animals to nanoscale particulates via intratracheal instillation. The preparation of the nanoscale test material is likely derived from bulk sources. Alternatively, it was acknowledged that, as a screening tool, the results gained from instillation studies generally are predictive of inhaled, particle-related lung toxicity. Another alternate route of exposure is the pharyngeal aspiration technique, although this methodology requires validation.
- One advantage of the instillation (or pharyngeal) methodology vs. inhalation is that multiple forms of a nanomaterial-type can be tested. This reduces the expense and complexity of such testing, while preserving limited test material that may be required for a full inhalation exposure regimen.
- A consensus opinion among the group was that *in vitro* testing should be reserved only for mechanistic studies and should not be used for toxicity rankings. In this regard, *in vitro* "toxicity" studies must be validated (with *in vivo* findings) before the results can stand alone as a component of a tiered approach.
- Concerns were raised that the particle characteristics (e.g., aggregation dynamics) could be altered if the particles are prepared in solution for an instillation study, and this could

be significantly different from the particle characteristics when compared to an aerosol generation study. Thus, the question of formulating materials for instillation should be considered prior to undertaking an *in vivo* study.

- The group recognized that the dispersion of particles can profoundly affect both *in vitro* and/or *in vivo* results, and therefore inclusion of surfactant in the instillation carrier (vehicle) might be beneficial. While this combined preparation would not simulate a normal delivery (exposure), it may better approximate the interaction of inhaled particles with lung lining fluids following deposition.
- The group acknowledged that any prepared suspension of particles (for instillation studies) likely will modify the surface chemistry of the instilled particles.

What are the representative nanomaterials for testing? Please consider both the responses to question 1 above, and which nanomaterials are most likely to result in high worker or consumer exposure in the near future. Are carbon nanotubes representative of spherical nanoparticulates? Which nanoparticles can best be represented by spherically shaped particles?

The group considered that the initial choices should be based on the likelihood of exposure in the workplace or the R&D/academic laboratory. In this regard, it will be important to accurately characterize the occupational exposures to determine the appropriate form and dose(metric) of the nanoparticulate type(s) of interest. This may not be practical for new materials (for which assessment techniques are unavailable). Moreover, the methodologies for conducting aerosol exposure assessments for a variety of currently produced nanoparticulate types, including carbon nanotubes, are either very limited or need to be developed. It was suggested that carbon black particles might be a good starting point for developing aerosol exposure methodologies and determining appropriate metrics (i.e., mass, surface area, particle number).

As discussed above, much of the criteria for testing representative nanomaterials are predicated on the development of accurate exposure methodologies, because the information obtained from exposure assessments will dictate the source of testing material, and perhaps may be useful for delineating between the characteristics in the bulk synthesis and the nanoscale material to be tested. Given the limited database on nanomaterials, it would be prudent to design studies in conjunction with all available data; NIOSH is developing a useful website which may be a source of important information. Additional comments related to representative nanomaterials for testing are included below:

- It is suggested that the criteria for the initial nanoscale compounds for testing should be those particulates that are near commercialization – perhaps concomitant with the selected materials that some governmental agencies such as NIOSH or NTP may be testing (e.g., metal oxides, quantum dots, carbon nanotubes, fullerenes). In addition, positive and negative particulate benchmarks, such as crystalline silica and titanium dioxide should be included as reference materials.
- Each class of nanomaterials has different properties, and within classes there are likely to be heterogeneous responses.
- There is likely to be great variability among batches of nanomaterials used for aerosol generation studies of nanoparticulates (different degrees of particle aggregation). There

may be less variability in colloidal synthesis of nanoscale particulates.

- Given the variability of samples, even within "standardized" materials, it will be important to develop detailed particle characterization information prior to commencing a study. Perhaps NIST standards can be utilized if they are available or appropriate.

What are the short-term, intermediate-term, and long-term endpoints that should be of principal concern?

The group had limited time to consider this question. Some limited comments/suggestions are listed below:

- Given the current interest in the effects of particulate matter (PM), and the recent studies suggesting that inhaled particles translocate to the brain, clearly the cardiovascular and central nervous systems should be evaluated in intermediate or long-term studies.
- Histopathological evaluations may not be sufficiently sensitive for assessing adverse endpoints. Perhaps histopathology should be considered in conjunction with functional endpoints.
- For assessing extrapulmonary effects in subchronic inhalation studies, it was suggested that full pathology be utilized (e.g., evaluating the effects in organs such as the brain, heart and liver).
- It may be useful to assess and compare the effects of the same nanoscale materials by different routes of exposure (e.g., assessing intratracheal instillation vs. inhalation exposures).

Group 8: Issues in Assessing Dermal Uptake and Toxicity of Nanoscale Materials

Steve Roberts, University of Florida *(Moderator)*
CM Jenkins, United States Air Force/University of Florida *(Recorder)*

What test system should be used? In vitro skin penetration or in vivo? If in vivo, what animal model (e.g., mouse, rat, pig, human)? Should the in vivo model be hairless or haired skin?

The point of these questions is to ask whether there are unique considerations in choice of model systems when evaluating dermal uptake and toxicity of nanoscale materials. After considerable discussion, it was the conclusion of the group that considerations regarding choice of model (species; *in vitro* versus *in vivo*; etc.) are the same for all dermal studies, whether involving drugs, chemicals, or nanoscale materials. There are strengths and weaknesses associated with the use of different models, and the choice of model will depend upon the specific objectives of the study and the research question to be answered. No special issues were identified with respect to nanoscale materials that would lead to different choices in models. However, the group noted that the pathways of entry of nanoscale materials through the skin (e.g., through hair follicles and sweat glands) are largely unknown. As information on pathways is discovered, it may become an important consideration in the choice of experimental model.

In what form should nanostructures be used in dermal permeability and toxicity studies?

The most appropriate form of the nanostructures (e.g., aggregated versus dispersed) to be used will depend upon the objectives of the study. To develop information most relevant to human exposures, it is important to duplicate, as closely as possible, the form(s) of nanostructures under actual exposure conditions. For research purposes, it may be important to understand the influence of form on dermal penetration and toxicity. In this situation, manipulation of the form (e.g., preventing aggregation or actively disaggregating nanostructures) may be important in testing hypotheses.

What vehicles should be used?

As a general principle, the vehicle used in a study can significantly affect the dermal permeability of the material. As such, the choice of the vehicle must be relevant to the objective(s) of the study and clearly described. In many respects, the choice of vehicle for studies of nanoscale materials will be dictated by the same considerations that apply to other materials. However, as discussed elsewhere in the workshop report, characterization of nanoscale materials is particularly important, and this should include characterization in the vehicle.

What information about detection methodology and findings should be presented in a research report?

Several essential elements regarding detection methodology and findings were identified: 1) The method of detection should be identified in the article; 2) The limit of detection (LOD) should be specified, as well as the method of determining the LOD. It was noted during discussion that the ability to conduct studies *in vivo* is limited by the ability to detect absorbed nanoscale particles; and 3) As appropriate, the location of absorbed nanoparticles in the skin should be determined.

What is the appropriate metric for nanoscale materials in skin?

Concentration of a nanoscale material in skin can be expressed by a number of different metrics, such as mass, particle surface area or particle number per unit area of skin. At this point, there is no clear understanding of the best metric for dermal studies. It is likely that there will be no single best concentration metric; the most appropriate metric will depend upon the material.

As understanding of dose metrics increases, it is possible that concepts regarding the best choice of dose metric will change. Consequently, the test material should be sufficiently well characterized and described in research reports to permit interconversion between the metrics.

What about using surrogate reporter particles with enhanced detection?

External tagging of nanoscale materials can alter their behavior and compromise the predictive value of the experiment. Internal tagging may be useful (e.g., silica particles containing fluorescent dye), but the surrogate should have the same external properties as the material of interest.

Group 9: Detection and Quantification of Nanoscale Materials in Toxicity Studies

Vicki Colvin, Rice University *(Moderator)*
Greg Erdos, University of Florida *(Recorder)*

What are the technologies for detection and quantification of particles in environmental samples (e.g. air, water ...)?

The group interpreted this question to refer to the detection methods for particle counting, and not the broader issue of measuring the nanoparticle physical and chemical characteristics. In all media, the technologies for detection must first collect, concentrate, or extract nanoparticles, and second detect their presence. A key sampling issue is how can you be sure that you are detecting all of the nanoparticles and have good statistics?

In air (e.g., monitoring in a laboratory environment), one of the technologies is to charge particles and then sweep them onto a grid for TEM. Another technology is differential mobility analysis (DMA), which provides real time nanoparticle counting and size distribution. The lower limit of particles detected is 2 to 3 nm, with the potential to go to somewhat smaller particles. DMA provides number density, but no chemical or shape information. There may be the potential to combine DMA with mass spectrometry for more definitive information. DMA may also be combined with charged diffusion mobility for surface area. Additional technologies include the ion cyclotron and staged filtration.

Technologies for nanoparticle detection in water include chromatography, using refractive index or dynamic light scattering to indicate the presence and concentration of nanoparticles; centrifugation followed by TEM; filtration; high salt aggregation; dynamic light scattering without chromatography; and elemental specific methods that have been developed in trace element chemistry.

Extraction poses a challenge to detection of nanoparticles in soils, because the particles have strong adsorption tendency.

The group agreed that the technologies for detection of nanoparticles were most developed for air sampling, but that water issues were becoming more critical.

What are methods for detecting nanoparticles in tissues?

In biological media that are liquids, the methods include scanning probes, cryo-TEM, light scattering (for particles larger than 50 nm), MRI, scanning superconducting quantum interference device (SQUID) microscopy, and two-photon and near field confocal imaging.

Detection of nanoparticles in solid tissue samples requires appropriate sample preparation. This can be followed by several methods. Laser scanning fluorescence can be used if the particle has

been fluorescently tagged. TEM is widely applicable, but minimum particle size depends on the material (100 nm for carbon, 3-10 nm for gold). SEM is limited to nanoparticles that are 100 nm or larger. Elemental analysis can be applied by digesting away extraneous material, collecting the nanoparticles by centrifugation, digesting the particles, and then measuring their quantity by elemental analysis. Bio-Raman can be applied to detect nanoparticles in cells. Radiolabeling and other tagging schemes are additional possibilities.

The group considered the potential for non-destructive detection of nanoparticles in living tissue. This would be very valuable for imaging the fate of nanoparticles in cells and also in organs. Optical and magnetic imaging techniques make this possible; however, they are not general for any particle type. These methods, where applicable, are quantitative enough to track clearance, and mass balance can be used to determine fate.

Does decoration of a nanoparticle with a tag (e.g., fluorophore, radioactive element) change the particle?

The group noted that any decoration changes the particle. Specific concerns with fluorophores are whether a tag would stay connected to a particle, and also how the chemical properties might change. If molecular tag is greater than 10% of the nanoparticle size, issues become significant. Radioactive labels with the same elemental composition as the particle are better, but can be difficult to realize.

How can/should we characterize nanoparticles after sonicating them?

Sonication changes the chemistry of particles and, in fact, can be thought of as a synthesis method. Thus, it is best to repeat all characterization after sonication. Particles that are dispersed by sonication are often not stable, and aggregate with time. This makes characterization and reproduction of results difficult. With appropriate surface coatings, nanoparticles can be stable in solution for months. Related to the question of sonication is the situation where nanoparticles settle during the course of a test. In this case, there is a question as to whether the results reflect cell-nanoparticle interactions or are, in fact, more representative of two-dimensional surface interactions between cells and settled nanoparticles.

Group 10: Laboratory Safety and Disposal Issues

Andrew Maynard, National Institute for Occupational Safety and Health *(Moderator)*
Janet Carter, Procter and Gamble *(Recorder)*

What are the key exposure metrics that exposures should be measured against?

Following on from other discussions at the meeting regarding nanomaterial characteristics relevant to toxicity and health impact, the importance of understanding exposures with respect to mass, number and surface area concentration was emphasized. In addition, discussions highlighted the need to understand the nanostructure of materials, particularly when nanostructure has the potential to be biologically available. The size of discrete nanomaterial

particles was also considered an important characteristic. In the case of the latter, the need to relate particle size to physiologically significant interactions was noted, including respiratory tract deposition probability, deposition distribution, and translocation following deposition, clearance/retention and dermal penetration. The need to relate particle size to physical and chemical properties was noted.

Four specific criteria were identified as being relevant to nanomaterial exposure in relation to specific exposure metrics:

- Chemical characteristics (both bulk and surface)
- Biopersistence and bio-durability
- Aggregation/disaggregation potential
- Material dispersion

These criteria were considered important for all suspended nanomaterials, irrespective of whether the suspension medium was liquid or gas. For both media, exposure mechanisms and routes exist enabling the material to enter the body.

Do relevant measurement methods exist? Where are the information gaps? Are there potential technologies that can be developed?

Measurement methods for nanomaterial exposure were considered in relation to fundamental bulk material metrics such as mass, surface area and number concentration, as well as more general considerations such as particle size, particle chemistry (bulk and surface) and sampling methodology.

Sampling methodology. It was noted that standard protocols exist for taking occupational aerosol samples. Generally, personal sampling is preferred to sampling at a static location to ensure that samples represent the material being inhaled. However, it was acknowledged that personal sampling is an ideal that may not be achievable in many cases with methods for characterizing nanomaterial exposure.

Standard approaches to aerosol sampling cover off-line and on-line analysis. Off-line analysis is typified by filter samples that are analyzed after collection using gravimetric analysis, chemical analysis, and sometimes microscopic analysis. Off-line analysis is usually used to provide information on time-weighted average exposures, particularly when evaluating exposures against exposure limits. On-line analysis relies on instruments capable of providing a rapid response to materials. This approach offers a number of advantages over off-line analysis, including analysis of temporal variations in exposure and monitoring process-specific exposures.

Sampling methodologies for other exposure routes were not discussed.

Particle size. Although nanoparticle size is clearly important in determining dose to specific target organs and subsequent impact, it is currently uncertain whether there are clear particle size ranges that should be measured when evaluating nanomaterial exposure.

For instance, toxicology data are currently insufficient to determine whether exposure to materials with the same specific surface area but different mean particle diameters will elicit different biological responses.

Some discussion was held on the measurement of particle size. No one in the group was aware of any personal sampling devices that enable on-line measurement of aerosol size distribution from diameters of a few nanometers upwards, although it was noted that technologies exist that could be adapted to develop appropriate personal samplers (including mobility analysis). *The development of personal aerosol size distribution measurement instruments was identified by the breakout group as a key R&D gap.*

Personal filter samples can be used for off-line analysis of particle size distribution using electron microscopy, and the same approach is applicable to static sampling. For particles above approximately 300 nm in diameter, portable Optical Particle Sizers (OPS) may be used to measure aerosol size distribution. If a portable OPS is used in parallel with a portable Condensation Particle Counter (CPC), additional information on particle concentration between the lower limit of the CPC (10 nm – 20 nm) and the lower limit of the OPS can be obtained. A number of static instruments are available that provide high resolution particle size distributions from a few nanometers upwards. These generally rely on differential mobility analysis below approximately 800 nm.

Chemical Composition (surface and bulk). It was noted within discussions that a number of off-line techniques are available that allow the bulk and surface analysis of collected material. However, no one within the group was aware of portable devices capable of providing relevant chemical information on nanomaterial exposures. The viability of applying mini-mass spectrometers to characterize the chemistry of nanoparticles in the field was raised. In addition, it was noted that on-line chemical speciation techniques such as aerodynamic time of flight mass spectrometry (ATOFMS) are potentially capable of providing information on nanoparticles *in situ*. It was also noted that other technologies are available that could be used for *in situ* nanoparticle chemical speciation.

Surface area. The conventional method for measuring aerosol surface area off-line is the Brunauer Emmett and Teller (BET) technique. However, there was some concern for how applicable this technique is to aerosol samples of nanomaterials, particularly where very low masses of material are collected. Portable methods are available that allow the *active* surface area of an aerosol to be measured, such as the DC2000CE diffusion charger from EcoChem (USA). However, *active* surface area only correlates linearly with geometric surface area for particles smaller than typically 100 nm in diameter, and it is currently unclear how biologically valid such an exposure measurement would be. Other methods for estimating surface area exposure have been proposed, including the use of size distribution measurements, derivation from mass measurements if specific surface area is known, and estimation from simultaneous number and mass concentration measurements. *The group identified the development of viable technologies for aerosol surface area monitoring as an R&D gap.*

Mass concentration. It was noted within discussions that measuring nanomaterial exposure in terms of mass concentration would tie in with current exposure monitoring methodologies. However there was concern over the appropriateness of the exposure metric, and whether gravimetric methods in particular are sufficiently sensitive to detect biologically significant concentrations of material. Although personal samplers for sub-100 nm diameter particles are not available, the technologies to develop such samplers exist. However, going back to the discussion on particle size, it is not certain that nanomaterial exposure should be limited to particles smaller than 100 nm.

Number concentration. Portable particle counters (CPCs) are available that measure the number concentration of particles from approximately 10 nm in diameter up to typically 1 – 10 μm in diameter. There was some discussion as to whether CPCs could or should be developed into personal sampling devices.

How effective are current controls and personal protective equipment?

The issue of how to determine appropriate levels of nanomaterial containment was discussed by the group. While it was acknowledged that data are too sparse for clear recommendations, it was also clear that there are likely to be different criteria for R&D environments and for commercial production/scale-up environments. There was agreement that a viable approach for R&D environments is to control exposures to be as low as reasonably practicable. For commercial production/scale-up, it was acknowledged that such a stringent approach may not always be viable, and that a different set of criteria will be needed.

Regarding Personal Protective Equipment (PPE), a number of unanswered questions were raised on how effective current PPE is in protecting against nanomaterial exposures, how PPE effectiveness should be measured if the hazard associated with a nanomaterial is unknown, and how appropriate levels of PE should be selected. In the absence of quantitative information, it was agreed that a good approach is to start with what is known (standard hygiene procedures; expected glove, clothing and respirator performance; etc.), and to move on from this position as new information becomes available. *R&D Gaps identified include: 1) The need for R&D demonstrating the effectiveness of PPE and engineering controls for nanomaterials; 2) Development of a database of information regarding available technologies and/or approaches to controlling nanomaterial exposures; 3) Development of Material Safety Data Sheets (MSDS) as an effective tool for informing users of nanomaterial hazards and handling procedures; and 4) Development of alternative risk management approaches such as control banding.*

How should nanomaterials be disposed of, and spills cleaned up?

Discussions revealed that very little is currently known about appropriate ways of cleaning up nanomaterial spills and disposing of nanomaterials in an R&D environment. However, it was acknowledged that there is an urgent need for basic information in this area. Specific questions raised included whether standard clean-up and disposal methods are appropriate for nanomaterials, whether new approaches need to be developed, and whether nanomaterial 'denaturing' is relevant to addressing large-scale disposal and potential release. *The group identified development of information and guidelines on the safe clean up and disposal of*

nanomaterials in R&D environments as an R&D gap.

Appendix A: Agenda

Developing Experimental Approaches for the Evaluation of Toxicological Interactions of Nanoscale Materials

November 3-4, 2004
University of Florida Hotel and Conference Center
1714 SW 34th Street
Gainesville, Florida 32607

Tuesday, November 2, 2004

7:00 – 8:30 PM	**Welcome Reception** (Century Ballroom B)

Wednesday, November 3, 2004

07:00–05:30 PM	**Registration and Attendee Services** (Conference Registration Desk)
07:30–08:30 AM	**Continental Breakfast** (Break Pavilion)
08:30–08:45 AM	**Welcoming Remarks** (Century Ballroom B & C)
08:45–09:15 AM	**"Nanotechnology development and potential toxicological issues."** **Clayton Teague**, National Nanotechnology Coordination Office
09:15–09:45 AM	**"Characterization of nanoscale structures."** **Brij Moudgil**, University of Florida
09:45–10:15 AM	**BREAK** (Break Pavilion) *Moderator/Recorder Meeting* (Century B & C)
10:15–10:45 AM	**"Dosimetry issues and challenges in characterizing the fate of nanoscale materials in the body."** **Günter Oberdörster**, Rochester University

Wednesday, November 3rd (Continued)

10:45–11:15 AM	**"Challenges in the design of toxicity studies of nanoscale materials."** **Michael Luster**, National Institute for Occupational Safety and Health
11:15–11:45 AM	**Charge to Breakout Groups**
11:45–01:00 PM	**LUNCH** (Albert's Restaurant)
01:30–03:00 PM	**Breakout Session I** Group 1: "Characterization of Nanoscale Materials" (Magnolia) Group 2: "Dosimetry of Nanoscale Materials" (Cedar) Group 3: "Nanoscale Materials Testing Protocols – Group A" (Birch) Group 4: "Nanoscale Materials Testing Protocols – Group B" (Azalea) Group 5: "Exposure Protocols: Practical Issues" (Hawthorne)
03:00-03:30 PM	**BREAK** (Break Pavilion)
03:30–05:00 PM	**Breakout Session II** Group 6: "Issues in Assessing Potential Immune System Effects" (Magnolia) Group 7: "Issues in Assessing Pulmonary Intake and Toxicity of Nanoscale Materials" (Cedar) Group 8: "Issues in Assessing Dermal Uptake and Toxicity of Nanoscale Materials" (Birch) Group 9: "Detection and Quantification of Nanoscale Materials in Toxicity Studies" (Azalea) Group 10: "Laboratory Safety and Disposal Issues" (Hawthorne)
05:00–05:30 PM	**Moderator/Recorder Meeting** (Hickory)
06:30–08:00 PM	**DINNER** (Albert's Restaurant)

Thursday, November 4th

07:00-12:00 PM	**Registration and Attendee Services** (Conference Registration Desk)
07:30-08:30 AM	**Continental Breakfast** (Break Pavilion)
08:30–12:00 PM	**Breakout Group Reports & Discussion** (Century Ballroom B & C)
12:00–01:00 PM	**LUNCH** (Dogwood Lane)
01:00–02:30 PM	**Breakout Group Reports & Discussion** (Century Ballroom B & C)
02:30–03:00 PM	**Closing Remarks & "Next Steps"** **John Bucher**, National Institute of Environmental Health Sciences

Appendix B: List of Participants

James Baker, University of Michigan, Breakout Groups 2 and 6
John Balbus, Environmental Defense, Breakout Groups 3 and 10
Mark Banaszak-Holl, University of Michigan, Breakout Groups 1 and 9
David Barber, University of Florida, Breakout Groups 3 and 7
Karen Blackburn, Procter and Gamble, Breakout Groups 3 and 10
Robert Bronaugh, Center for Food Safety and Applied Nutrition/US FDA, Breakouts 5 and 8
Stanley Brown, Center for Devices and Radiological Health/US FDA, Breakout Groups 1 and 9
Scott Brown, University of Florida, Student participant
John Bucher, National Institute of Environmental Health Sciences, Breakout Group 1
Janet Carter, Procter and Gamble, Breakout Groups 4 and 10
Mengdawn Cheng, Oak Ridge National Lab, Breakout Groups 5 and 9
Vicki Colvin, Rice University, Breakout Groups 1 and 9
Michael Cunningham, National Institute of Environmental Health Sciences, Breakouts 2 and 9
Raymond David, Eastman Kodak Company, Breakout Groups 4 and 8
Donn Dennis, University of Florida, Breakout Groups 5 and 9
Nancy Denslow, University of Florida, Breakout Groups 4 and 6
Kevin Dreher, United States Environmental Protection Agency, Breakout Groups 4 and 7
Debamitra Dutta, University of Florida, Student participant
Greg Erdos, University of Florida, Breakout Groups 5 and 9
Richard Flagan, California Institute of Technology, Breakout Groups 2 and 7
Jeffry Gearhart, Air Force Research Laboratory, Human Effectiveness Division, Groups 2 and 9
Dori Germolec, National Institute of Environmental Health Services, Breakout Groups 5 and 6
Peter Greaney, WorkCare, Breakout Groups 3 and 10
Krista Hess, Air Force Research Laboratory, Human Effectiveness Division, Breakouts 5 and 8
Angela Hight Walker, National Institute of Standards and Technology, Breakout Groups 1 and 9
Paul Howard, National Center for Toxicological Research/US FDA, Breakout Groups 2 and 8
Matthew Hull, Luna Innovations, Breakout Groups 4 and 10
Saber Hussain, Air Force Research Laboratory, Human Effectiveness Division, Breakout 9
Mike Jenkins, United States Air Force, Breakout Groups 1 and 8
Shane Journeay, University of Saskatchewan, Student participant
Valerian Kagan, University of Pittsburgh, Breakout Groups 5 and 6
Agnes Kane, Brown University, Breakout Groups 5 and 7
Barbara Karn, United States Environmental Protection Agency, Breakout Groups 4 and 10
Chong Kim, United States Environmental Protection Agency, Breakout Groups 2 and 7
Ben Koopman, University of Florida, Breakout Groups 1 and 9
Walter Kozumbo, Air Force Office of Scientific Research, Breakout Groups 3 and 10
Vijay Krishna, University of Florida, Student participant
Timothy Landry, Dow Chemical Company, Breakout Groups 2 and 7
Ken Loewen, Consumer Product Safety Bureau, Health Canada, Breakout Groups 4 and 10
Michael Luster, National Institute for Occupational Safety and Health, Breakout Groups 4 and 6
Scott Masten, National Institute of Environmental Health Sciences, Breakout Group 4
Brian Mayes, General Electric, Breakout Groups 4 and 10
Andrew Maynard, National Institute for Occupational Safety and Health, Breakouts 1 and 10
Wilfred McCain, US Army Center for Health Promotion and Preventive Medicine, 3 and 10

Jake McDonald, Lovelace Respiratory Research Institute, Breakout Groups 5 and 7
Nancy Monteiro-Riviere, North Carolina State University, Breakout Groups 5 and 8
David Moraga, University of Florida, Breakout Groups 5 and 6
Brij Moudgil, University of Florida, Breakout Group 1
Vladimir Murashov, National Institute for Occupational Safety and Health, Breakouts 3and 8
Günter Oberdörster, University of Rochester, Breakout Groups 2 and 7
Maria Palazuelos, University of Florida, Student participant
Charlie Pennington, Luna Innovations, Breakout Groups 1 and 10
Raymond Pieters, Utrecht University, Breakout Groups 5 and 6
Kevin Powers, University of Florida, Breakout Group 1
Steve Roberts, University of Florida, Breakout Groups 5 and 8
Nakissa Sadrieh, Center for Drug Evaluation and Research/US FDA, Breakout Groups 3 and 8
Annette Santamaria, Exponent, Breakout Groups 4 and 7
Swadeshmukul Santra, University of Florida, Breakout Groups 1 and 9
Katherine Sarlo, Procter and Gamble, Breakout Groups 2 and 6
Nora Savage, United States Environmental Protection Agency, Breakout Groups 5 and 6
Phil Sayre, United States Environmental Protection Agency, Breakout Groups 3 and 8
John Schlager, United States Air Force, Breakout Groups 5 and 6
Anna Shvedova, National Institute for Occupational Safety and Health, Breakout Groups 5 and 7
Wolfgang Sigmund, University of Florida, Breakout Groups 1 and 9
Cynthia Smith, National Institute of Environmental Health Sciences, Breakout Groups 1 and 7
Anita Street, United States Environmental Protection Agency, Breakout Groups 4 and 9
Susan Sumner, RTI International, Breakout Groups 3 and 7
Clayton Teague, National Nanotechnology Coordination Office, Breakout Groups 3 and 10
Karluss Thomas, ILSI Health and Environmental Sciences Institute, Breakout Groups 4 and 10
Sally Tinkle, National Institute of Environmental Health Sciences, Breakout Groups 2 and 8
Mark Torasson, National Institute for Occupational Safety and Health, Breakouts 3 and 10
Ron Turco, Purdue University, Breakout Groups 5 and 9
Ann Tveit, Arkema, Inc., Breakout Groups 3 and 10
Nigel Walker, National Institute of Environmental Health Sciences, Breakout Group 3
William Wallace, National Institute for Occupational Safety and Health, Breakouts 2 and 9
David Warheit, Dupont, Breakout Groups 2 and 7
Tian Xia, UCLA, Breakout Groups 5 and 6

Final Fiscal Report

Funding in the amount of $8333.00 was used to support the workshop "Developing Experimental Approaches for the Evaluation of Toxicological Interaction of Nanoscale Materials".

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